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(54) OLIGONUCLEOTIDES USED FOR DETECTING VIBRIO PARAHAEMOLYTICUS AND METHOD OF DETECTION THEREWITH

(57) An oligonucleotide having a nucleic acid sequence derived from SEQ ID NO:1 and at least one site capable of amplifying a nucleic acid sequence characteristic of *Vibrio parahaemolyticus* ; the above oligonucleotide having a nucleic acid sequence unavailable from SEQ ID NO:3; the above oligonucleotide incapable of amplifying nucleic acid sequences originating in *Vibrio alginolyticus* and *Vibrio harvei* ; the above oligonucleotide represented by the sequence of CGG CGT GGG TGT TTC GGT AGT or TCC GCT TCG CGC TCA TCA ATA; and a method of detecting *Vibrio parahaemolyticus* by preparing a primer set comprising two of the above oligonucleotides, selectively amplifying therewith a DNA gyrase subunit B gene sequence contained in a specimen as a target, and determining whether or not there is a gyrB unit specific for *Vibrio parahaemolyticus* in the specimen. This method has made it possible to provide a primer which specifically reacts with a gyrB gene of *Vibrio parahaemolyticus* to thereby differentiate and identify the same among other vibrios and strains other than the genus *Vibrio* . The primer specific for *Vibrio parahaemolyticus* serves to detect 285-bp gyrB gene fragments specific for this vibrio by the PCR method without the necessity for DNA extraction or like operations from bacterial cells.

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Description

FIELD OF THE INVENTION

[0001] This invention relates to oligonucleotide primers for amplification of the target nucleotide sequence characteristic of *Vibrio parahaemolyticus* (abbreviated as "VP" somewhere hereinafter). This invention relates to the method for detecting *Vibrio parahaemolyticus* based on the polymerase chain reaction (PCR) using a primer specific for the DNA gyrase sub-unit B gene (Nucleotide sequence of DNA gyrase B subunit, abbreviated as "*gyrB*" hereinafter).

PRIOR ART

[0002] *Vibrio parahaemolyticus* is known to cause food poisoning in many countries. It is found not only in the intestine but also in other organs and in the postoperative wound. *Vibrio parahaemolyticus* is a Gram negative, polymorphic, bacilliform, halophilic, facultative anaerobe, which ferments carbohydrate to generate gas. It forms green colonies on thiosulfate-citrate-bile-sucrose (TCBS) agar.

[0003] For detection of *Vibrio parahaemolyticus*, is used usually a method where the specimen is cultivated in an enrichment medium followed by isolation by the selective plate culture. The conventional method of detection requires one week, and therefore a more rapid method has been desired.

[0004] The fluorescence assay based on determination of trypsin activity can detect rapidly *Vibrio parahaemolyticus* but cannot differentiate *Vibrio parahaemolyticus* from *Vibrio alginolyticus* or *Vibrio harvei*. The conventional methods for identification of *Vibrio parahaemolyticus* and *Vitrio alginolyticus* are time-consuming because the 16S rRNA sequence reveals homology of 99.7% between *Vibrio parahaemolyticus* and *Vitrio alginolyticus*.

[0005] The genus *Vibrio* includes 37 different species, all of which are derived from aquatic environment. Based on the systematic data of rRNA, species known as *V. angullarum*, *V. ordalli*, and *V. damsela* have been newly classified as *Listonella* or *Photobacterium*. Ten species are involved in gastroenteritis, infection of the wound, and human septicemia, while 7 species are known to be pathogens for fish. *Vibrio parahaemolyticus* occurs usually in an environment such as river-mouth and sea, being isolated from sea water and fishes and shellfishes often in summer.

[0006] For isolation and identification of *Vibrio parahaemolyticus*, the specimen is inoculated into a selective medium such as the bromothymolblue-teepol agar medium or TCBS agar medium, followed by isolation of bluish green colonies and examinations for the biochemical properties of the colonies. Unfortunately many *Vibrio* species show the same responses, and thus more detailed biochemical examinations are required for reliable identification. Examinations for a variety of biochemical properties on many isolates are time-consuming and laborious. Serological identification of *Vibrio parahaemolyticus* shows a cross reaction with other *Vibrio* species.

[0007] A method for identification of a *Vibrio* species was developed which used DNA. In this method were used DNA probes capable of amplifying the cholera toxin operon from *V. cholera* 01 to identify specifically the bacterium. These probes cross-react with *Vibrio* species other than cholera toxin-producing *V. cholera*. A method for identification of *V. vulnificus* has been developed in which hybridization is carried out on a membrane filter by using a fluorescent-labeled oligonucleotide probe (Wright, A.C. et al., Appl. Environ. Microbiol. 59: 541-546, 1993).

[0008] In addition, the oligonucleotide DNA probe was constructed from a portion of the cytolysin gene (*hlyA*) sequence of *V. vulnificus* and labeled through the covalent bond. These probes do not react with non-toxinogenic *V. vulnificus* and therefore do not detect all strains of *V. vulnificus*.

[0009] Similarly, other molecular biologic methods using the toxic factor (TDH, TRH) genes as the target can detect toxinogenic *V. parahaemolyticus*, though based on the toxic factor, all strains of *V. parahaemolyticus* cannot be detected.

DISCLOSUR OF THE INVENTION

[0010] The object of this invention is to provide a method for differentiation of *Vibrio parahaemolyticus* from other 36 *Vibrio* species.

[0011] The object of this invention is to provide a method for detection of the 285-bp *gyrB* gene fragments specific for *Vibrio parahaemolyticus* by the PCR method without the necessity for DNA extraction or like operations from bacterial cells by use of *Vibrio parahaemolyticus*-specific primers.

[0012] Because this invention provides oligonucleotide probes useful for PCR, this invention relates to oligonucleotide primers for amplification of the target nucleotide sequence characteristic of *Vibrio parahaemolyticus*. The primers are exemplified by Sequences No.5 and No.6, which are used as a primer set for detection of the target nucleotide sequence characteristic of *Vibrio parahaemolyticus*.

[0013] The primer set is used in the method for determining whether or not there is the target nucleotide sequence characteristic of *Vibrio parahaemolyticus*. A *Vibrio parahaemolyticus*-specific primer is capable of detecting a *Vibrio*

parahaemolyticus-specific *gyrB* gene fragment by the PCR method.

[0014] In this invention "primer" means an oligonucleotide which is produced synthetically or biologically and includes a specific nucleotide sequence which permits hybridization to a section containing the target nucleotide sequence.

[0015] A primer is capable of replicating a full target nucleotide sequence by synthesis by extension in the presence of polymerase or an analogous enzyme.

[0016] A primer is used in the method for amplification of nucleotide sequence, such as PCR and sequence displacement amplification (SDA). A particular primer, especially those useful for SDA technology, contains not only the sequence capable of hybridization to the target nucleic acid, but also a recognition sequence for restriction endonuclease and an arbitrary sequence which allows polymerase or another enzyme continuing polymerase-like activity to direct itself to initiate the synthesis of the template-specific oligonucleotide.

[0017] In this invention, "hybridization" means a process where, under pre-determined reaction conditions, partially or completely complementary nucleic acid chains, standing opposite to each other in an anti-parallel way, form a two-strand nucleic acid through specific and stable hydrogen bonds.

[0018] As described above, this invention relates to oligonucleotide primers useful for determination of the presence or absence of the target nucleotide sequence that is specific for *Vibrio parahaemolyticus*.

[0019] The procedures used for such determination include not only the PCR-based gene amplification procedure, but also Southern hybridization, a prior art, wherein the primer is used as a probe.

[0020] The primer in this invention is specific for the *gyrB* subunit gene sequence of *Vibrio parahaemolyticus*. The probe is specific for the internal consensus sequence in the primer amplification product.

[0021] The inventors have studied a method using the *gyrB* gene encoding the B subunit protein of DNA gyrase (topoisomerase II) as a highly specific probe, to solve the problems in the prior art described above.

[0022] A method has been reported recently for detection and taxonomic analysis of *Pseudomonas putida* with a universal primer with which the *gyrB* gene was sequenced directly. Such universal primers were used for PCR-based amplification of the *gyrB* gene fragments of various Gram-negative and Gram-positive bacteria. The inventors used these existing primers to amplify the 1.2-kb *gyrB* fragments of 37 *Vibrio* species. The *gyrB* base sequence of *Vibrio parahaemolyticus* ATCC17802 and that of *Vibrio alginolyticus* ATCC17749 (abbreviated as 'VA' somewhere hereinafter) have been shown.

[0023] In addition, the inventors prepared PCR primers capable of amplifying and identifying only the *gyrB* gene of *Vibrio parahaemolyticus*. The sensitivity of these *Vibrio parahaemolyticus*-specific primers was investigated with 118 *Vibrio parahaemolyticus* strains, 20 *Vibrio alginolyticus* strains, and other 78 strains.

[0024] Yamamoto and Harayama (Appl. Environ. Microbiol. 61; 1104-1109, 1995) and others prepared PCR primers capable of amplifying the *gyrB* gene from two conserved regions of the amino acid sequences of the DNA gyrase subunit B proteins. These primers were used for amplification of the about 1.2-kb *gyrB* gene fragments from various bacteria.

[0025] The *gyrB* gene fragment amplified from *Vibrio parahaemolyticus* ATCC17802 and that from *Vibrio alginolyticus* AATCC17749 were cloned by using a suitable vector according to the conventional method of recombination (Sambrook et al., Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York. 1989).

[0026] Desirable vectors include pGEMzf(+), and any common vector may be used.

[0027] The 1.2 kb *gyrB* gene fragment from *Vibrio parahaemolyticus* cloned with pGEMzf(+) is called a plasmid pVP*gyrB*, and that from *Vibrio alginolyticus* a plasmid pVA*gyrB*.

[0028] The probe is amplified also with a conventional method (Sambrook et al., 1989). For example, a plasmid is inserted into the vector for transformation of *Escherichia coli* with an effective mean such as calcium chloride. Transformed cells are cultivated under appropriate conditions.

[0029] The target genes are collected after lysis of bacterial cells, and purified by the alkali method or the like. Purified plasmid is used as the specimen.

[0030] The PCR method was tried for detection and differentiation of *Vibrio parahaemolyticus* from other *Vibrio* species including *Vibrio alginolyticus*. This method is capable of amplifying a sequence homologous with the probe, practically increasing the sensitivity.

[0031] According to the base sequence of the probe, the synthesized oligonucleotide primer amplifies DNA only when the target base sequence is present in the specimen. Not only the sensitivity is enhanced but also an absolute specificity can be attained by using an oligonulcleotide having specificity based on the base sequence of the DNA probe.

[0032] For preparation of effective primers, the base sequence of pVP*gyrB* and that of pVA*gyrB* were determined with the DNA sequencer according to the conventional method.

[0033] For determination of the base sequence of *gyrB*, the base sequence of the N-terminal and C-terminal regions of the amplified fragment was also determined by using UP-1S and UP-2Sr primers (Yamamoto and Harayama, Appl. Environ. Microbiol. 61: 1104-1109, 1995). For extension of the determined base sequence, an additional primer was prepared from the base sequence determined by using UP-1S. The length of the whole base sequence of the amplified

fragment is 1258 bp, and the sequence is shown in Sequence No.1 (pVP*gyrB*) and Sequence No.3 (pVA*gyrB*). The amino acid sequence encoded by Sequence No.1 and that encoded by Sequence No.3 are shown in Sequence No.2 and Sequence No.4, respectively.

[0034] With this information of the base sequence, 21-bp primers were prepared that can detect and identify *Vibrio parahaemolyticus* from other bacteria. These primers contain Sequences No.5 and No.6, being usable as a primer set.

[0035] These novel primers are useful in the existing assay method using PCR (Saiki et al., Science 239: 487-491, 1988). These primers are used for amplification of the target DNA in a specimen, making the amount of DNA sufficient for detection. Following the amplification step, the step of detection may be performed by any method as far as it is effective for detection of DNA, for example, by electrophoresis on agarose gel.

[0036] The target DNA functions as the template. Amplification of the template DNA in the specimen is effected by treatment of the primer pair with a duplex DNA. This treatment results in extension of the sequence complementary to each nucleotide sequence. The resultant sequence functions as the template of the primer. The treatment process comprises denaturation of DNA, annealing to a sequence complementary to the primer, and extension of the primer with DNA polymerase (e.g. Taq polymerase), and is repeated until DNA has been produced in an amount sufficient for detection of the target sequence. The conditions of the amplification based on PCR are summarized in the Example 3 below.

[0037] Following amplification, the amplified sequence is detected by electrophoresis on agarose gel. The primer pair [VP1(Sequence No.5), VP2 (Sequence No.6)] amplifies the 285-bp when the *gyrB* gene sequence is used as the target.

[0038] The 285-bp DNA was amplified for 37 *Vibrio* strains obtained from ATCC, JCM, CDC, and NCIMB collections. The chromosomal DNA preparations from these bacteria were obtained according to the conventional method (Sambrook et al., Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, 1989). The target DNA, 1 μg, was subjected to PCR. The 285-bp specific band was found only in the preparation from *Vibrio parahaemolyticus*, while it was not detected in any preparation from other species (Table 1). However the PCR-based amplification using the primer set (UP-1, UP-2r) according to Yamamoto and Harayama (1995) detected 1.2-kb *gyrB* fragment, and thus could confirm the presence of the DNA gyrase B subunit. Based on these findings, it is concluded that the primers of this invention are *Vibrio parahaemolyticus*-specific and usable for detection of the pathogen.

[0039] This invention relates to the oligonucleotides characterized in that they have the nucleotide sequence derived from Sequence No.1 and include at least one site capable of amplifying the *Vibrio parahaemolyticus*-specific nucleotide sequence. This invention relates to the oligonucleotides characterized in that they have the nucleotide sequence derived from Sequence No.1 but not from Sequence No.3 and include at least one site capable of amplifying the *Vibrio parahaemolyticus*-specific nucleotide sequence.

Table 1

S.no.	Microbes	Strain Number	PCR results of gyrB	
			1.2-kb	285bp
1	*Vibrio aestuarianus*	ATCC35048	+	-
2	*Vibrio albensis*	ATCC14547	+	-
3	*Vibrio alginolyticus*	ATCC17749	+	-
4	*Vibrio campbelli*	ATCC25920	+	-
5	*Vibrio carchariae*	ATCC35084	+	-
6	*Vibrio cholerae* 01	P1418	+	-
7	*Vibrio cholerae non* 01	NR	+	-
8	*Vibrio cincinnatiensis*	ATCC35912	+	-
9	*Vibrio costicola*	ATCC33508	+	-
10	*Vibrio diazotrophicus*	ATCC33466	+	-
11	*Vibrio fischeri*	ATCC 7744	+	-
12	*Vibrio fluvialis*	JCM3752	+	-
13	*Vibrio furnissii*	ATCC35016	+	-

Table 1 (continued)

S.no.	Microbes	Strain Number	PCR results of gyrB	
			1.2-kb	285bp
14	*Vibrio gazogenes*	ATCC29988	+	-
15	*Vibrio harveyi*	ATCC14126	+	-
16	*Vibrio hollisae*	CDC75-80	+	-
17	*Vibrio logei*	ATCC29985	+	-
18	*Vibrio marinus*	ATCC15381	+	-
19	*Vibrio mediterranei*	ATCC43341	+	-
20	*Vibrio metschnikovii*	ATCC 7708	+	-
21	*Vibrio mimicus*	CNS9582	+	-
22	*Vibrio mytili*	NCIMB13275	+	-
23	*Vibrio natriegens*	ATCC14048	+	-
24	*Vibrio navarrensis*	NCIMB13120	+	-
25	*Vibrio nereis*	ATCC25917	+	-
26	*Vibrio nigripulchritudo*	ATCC27043	+	-
27	Vibrio ordalii	ATCC33509	+	-
28	*Vibrio orientalis*	ATCC33934	+	-
29	*Vibrio parahaemolyticus*	ATCC17802	+	+
30	*Vibrio proteolyticus*	ATCC15338	+	-
31	*Vibrio salmonicida*	ATCC43839	+	-
32	*Vibrio splendidus*	ATCC33125	+	-
33	*Vibrio tubiashii*	ATCC19109	+	-
34	*Vibrio vulnificus*	ATCC2046	+	-
35	*Listonella anguillarum*	ATCC19264	+	-
36	*Listonella pelagia*	ATCC25916	+	-
37	*Photobacterium damsela*	ATCC33539	+	-

ATCC : American Type Culture Collection
JCM : Japan Collections of Microrganisms
NCIMB : National Collections of Industrial and Marine Bacteria.
CDC : Centre for Disease Control

[0040] The symbols other than those described above refer to the strain names.

[0041] Also the standard strains of genera *Aeromonas*, *Alteromonas*, *Marinomonas*, *Shigella*, *Shewanella*, *Salmonella*, *Escherichia* and *Staphylococcus aureus* were examined for the presence of *gyrB* and the *Vibrio parahaemolyticus*-specific 285-bp. As shown in Table 2, *gyrB* was found to be present in all of the strains though the *Vibrio parahaemolyticus*-specific 285-bp amplification was found in none of the strains.

Table 2

S.no.	Microbes	Strain number	PCR results of gyrB	
			1.2-kb	285bp
1	*Alteromonas atlantica*	ATCC19262	+	-

Table 2 (continued)

S.no.	Microbes	Strain number	PCR results of gyrB	
			1.2-kb	285bp
2	*Alteromonas carrogeenovara*	ATCC43555	+	-
3	*Alteromonas citrea*	ATCC29719	+	-
4	*Alteromonas espejiana*	ATCC29659	+	-
5	*Alteromonas haloplanktis*	ATCC14393	+	-
6	*Alteromonas luteoviolaceae*	ATCC33492	+	-
7	*Alteromonas macleodii*	ATCC27126	+	-
8	*Alteromonas tetraodonis*	NCIMB13177	+	-
9	*Alteromonas undina*	ATCC29660	+	-
10	*Marinomonas communis*	ATCC27118	+	-
11	*Marinomonas vaga*	ATCC27119	+	-
12	*Aeromonas hydrophila*	ATCC19570	+	-
13	*Esherichia coli*	ATCC25922	+	-
14	*Salmonella typhimurium*	ATCC13311	+	-
15	*Shewanella putrefaciens*	ATCC 8071	+	-
16	*Shigella dysenteriae*	ATCC13313	+	-
17	*Shigella sonneii*	ATCC29930	+	-
18	*Staphylococcus aureus*	ATCC12600	+	-

[0042] Various phenotypes, serotypes, and toxinogenic types have been reported for *Vibrio parahaemolyticus*. A probe specific for the thermostable hemolysin, a toxin produced by *Vibrio parahaemolyticus*, has been reported (Nishibuchi et al., FEMS Microbiol. Lett. 55: 251-256, 1990). Such toxin-specific probes are clinically important, though they cannot detect all types of *Vibrio parahaemolyticus*. For prevention of contamination of food with *Vibrio parahaemolyticus*, it is essential to detect all types of *Vibrio parahaemolyticus* in food or in the environment. Then *Vibrio parahaemolyticus* strains isolated from food, water, soil, and other materials were investigated for their phenotypes, serotypes, and the toxicity. All of the 118 *Vibrio parahaemolyticus* strains were subjected to PCR using the primers VP1 and VP2. It was evident that the 285-bp had been amplified.

[0043] The results are summarized in Table 3. The strains shown in Table 3 are those isolated from and identified in food, soil, water or feces, some of which were kindly given by Prof. Shinoda, Okayama University, and Prof. Yamamoto, Kyushu University. As shown in Table 3, amplification of the 285-bp was not observed in any of the 20 strains of *Vibrio alginolyticus* also isolated from various materials.

[0044] This invention relates to oligonucleotides characterized in that they have the nucleotide sequence obtained from Sequence No.1, contain at least one site capable of amplifying the nucleotide sequence characteristic of *Vibrio parahaemolyticus*, and cannot amplify the nucleotide sequence derived from *Vibrio alginolyticus* or *Vibrio harvei*.

[0045] This invention relates to oligonucleotides characterized in that they have the nucleotide sequence obtained from Sequence No.1 but not from Sequence No.3, contain at least one site capable of amplifying the nucleotide sequence characteristic of *Vibrio parahaemolyticus*, and cannot amplify the nucleotide sequence derived from *Vibrio alginolyticus* or *Vibrio harvei*.

Table 3

S.no.	Name	Strain no.	Kanagawa phenomenon	PCR
1	*V.parahaemolyticus*	33-7	+	+
2	*V.parahaemolyticus*	33-8	+	+
3	*V.parahaemolyticus*	33-10	+	+

Table 3 (continued)

S.no.	Name	Strain no.	Kanagawa phenomenon	PCR
4	*V. parahaemolyticus*	V83	+	+
5	*V.parahaemolyticus*	WP-1(y)	+	+
6	*V.parahaemolyticus*	WP-1	+	+
7	*V.parahaemolyticus*	39-11	-	+
8	*V.parahaemolyticus*	46-11	-	+
9	*V. parahaemolyticus*	AQ3301	-	+
10	*V.parahaemolyticus*	AQ3314	-	+
11	*V. parahaemolyticus*	AQ3321	-	+
12	*V.parahaemolyticus*	AQ3326	-	+
13	*V.parahaemolyticus*	AQ3331	-	+
14	*V.parahaemolyticus*	AQ3343	-	+
15	*V.parahaemolyticus*	AQ3345	-	+
16	*V.parahaemolyticus*	AQ3346	-	+
17	*V.parahaemolyticus*	AQ3354	-	+
18	*V.parahaemolyticus*	AQ3360	-	+
19	*V.parahaemolyticus*	AQ3627	-	+
20	*V.parahaemolyticus*	AQ3629	-	+
21	*V.parahaemolyticus*	AQ3633	-	+
22	*V.parahaemolyticus*	AQ3634	-	+
23	*V.parahaemolyticus*	BB22	-	+
24	*V.parahaemolyticus*	ML34	-	+
25	*V.parahaemolyticus*	ML159	-	+
26	*V.parahaemolyticus*	ML1017	-	+
27	*V.parahaemolyticus*	MY67-6	-	+
28	*V.parahaemolyticus*	MY73-2	-	+
29	*V.parahaemolyticus*	OK80-480	-	+
30	*V.parahaemolyticus*	OKA80-214	-	+
31	*V.parahaemolyticus*	OKA80-232	-	+
32	*V.parahaemolyticus*	S53	-	+
33	*V.parahaemolyticus*	RIMD2210521	-	+
34	*V.parahaemolyticus*	AR1-01	-	+
35	*V.parahaemolyticus*	AR3-02	-	+
36	*V.parahaemolyticus*	AR4-01	-	+
37	*V.parahaemolyticus*	AR4-02	-	+
38	*V.parahaemolyticus*	AR6-01	-	+
39	*V.parahaemolyticus*	AR6-02	-	+
40	*V.parahaemolyticus*	AR7-01	-	+
41	*V.parahaemolyticus*	KB1-01	-	+

Table 3 (continued)

S.no.	Name	Strain no.	Kanagawa phenomenon	PCR
42	*V.parahaemolyticus*	KB1-03	-	+

[0046] For evaluation of the assay systems based on PCR, dilutions of the genomic DNA of *Vibrio parahaemolyticus* ATCC17802 strain were prepared and used as the template for PCR-based amplification. Even in the dilution containing only 1ng of the genomic DNA, detection was possible by amplification using the primers VP1 and VP2. For enhancement of the sensitivity from the ng level up to the pg level, DNA after electrophoresis was subjected to the Southern blotting. The dilution of cultivated *Vibrio parahaemolyticus* ATCC17802 cells was used for amplification with the primer described above, where the detection limit was about 1cfu/reaction tube. That is, the detection limit based on PCR was 1cfu/10 μ l or 10^3cfu/ml. Detection by plating or by use of a selective agar medium has a sensitivity capable of detecting one liable cell but requires much labor and much time.

[0047] The assay methods based on PCR are superior to the conventional detection methods from the viewpoint of the balance among the speed, sensitivity, and specificity which is essential for the method of detection of bacteria, and thus useful.

BEST EMBODIMENTS OF THE INVENTION

[0048] This invention is explained with Examples in the following. The Examples show the mode of working of the invention, and do not limit the invention at all.

Example 1

Conventional method of isolation of *Vibrio parahaemolyticus* in food

[0049] To 25 g of a food specimen, was added the alkaline peptone water [manufactured by Nissui Pharmaceutical Co., Ltd.] followed by inoculation with *Vibrio parahaemolyticus* ATCC17802 and incubation at 37°C for 18 hours. One loopful amount of the culture was inoculated into the TCBS agar medium by streaking and incubated at 37°C for 24 hours. All of green colonies were isolated further with the T_1N_1 agar medium (distilled water containing 1% of Bacto tryptone, 1% of NaCl, and 1.5% of agar). Sufficiently isolated colonies were subjected to biochemical examinations. The strains that showed the following properties were identified as typical *Vibrio parahaemolyticus*: positive tests for oxidase, lysine decarboxylase, ornithine decarboxylase, gellatinase, lipase, and chitinase; indole producing; viable at the salt concentration of 0.5 to 8% at 42°C; sensitive to O/129 (150 μ g); producing acids from glucose, mannitol, and mannose; negative tests for arginine dehydrogenase and arginase; not viable at salt concentration of 0%; and producing no acid from sucrose, lactose or salicin.

Example 2

Isolation of chromosomal DNA

[0050] A *Vibrio parahaemolyticus* strain was cultured by shaking in the TSB medium (manufactured by Eiken Chemical Co., Ltd.) at 37°C for about 24 hours. Cells were collected by centrifugation (manufactured by Tomy Seiko Co., Ltd.) (15,000 rpm, 15 minutes, 4°C), and suspended in 10 ml of sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Cells were lysed by lysozyme (final concentration 1 mg/ml; manufactured by Wako Pure Chemical Industries, Ltd.), and maintained at 37°C for 20 minutes while being shaken gently. To the cell lyzate was added SDS (final concentration 0.5%) and the mixture was incubated at 65 °C . For degradation of protein and RNA, were added proteinase K (final concentration 500 μ g/ml) and RNase (final concentration 5 μ g/ml), and the mixture was incubated at 37°C for 30 minutes and for 60 minutes, respectively. The samples were extracted three times with buffer saturated phenol (GIBCO/BRL), once with phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (24:1). Clear supernatant was obtained by centrifugation of the extract and DNA was precipitated by addition of two volumes of ice-cooled ethanol: 3M sodium acetate (10:1). The reaction mixture was kept overnight at -20°C. The DNA precipitate was concentrated by centrifugation, and ethanol was evaporated off under reduced pressure. The dried DNA was dissolved in the TE buffer, which was used as the DNA template. The purity of DNA was determined by electrophoresis on agarose gel, and the concentration of DNA was determined with the spectrophotometer.

Example 3

Assay based on PCR

Preparation of specimens

[0051] All cells including also those not used for extraction of DNA were used as the template. Fresh cells grown on agar media were used. Cells grown on liquid media were used after separation of cells by centrifugation followed by washing once with PBS buffer (pH 7.5), and a suitable number of resultant cells were used. In some cases, DNA extracted with phenol-chloroform was used as the template of PCR amplification. PCR amplification conditions

[0052] PCR assay was carried out with a DNA Thermal Cycler (Perkin Elmer Corp.). One hundred microliter of the reaction mixture (Tris-HCL 100 mM, $MgCl_2$ 15 mM, KCl 500 mM, pH 8.3) contains 100 ng of genomic DNA, 200 μ M of dNTPs, and 1 μ M of primer. DNA degeneration, annealing, and DNA extension were carried out at 94°C for 60 seconds, at 60°C for 60 seconds, and at 72°C for 120 seconds, respectively, and a total of 30 cycles of amplification were performed. Following amplification, detection was made by gel electrophoresis. Twenty microliter of the sample was subjected to electrophoresis on agarose gel (1% agarose, SeaKem ME, FMC Bioproducts, Rockland, Maine). The DNA band was stained with the ethidium bromide solution for 10 minutes and observed under ultraviolet irradiation.

INDUSTRIAL APPLICABILITY

[0053] A primer which reacts specifically with a *gyrB* gene of *Vibrio parahaemolyticus* to thereby differentiate and identify the same among other *Vibrios* and strains other than the genus *Vibrio* could be provided.

[0054] The *Vibrio parahaemolyticus*-specific primer serves to detect 285-bp *gyrB* gene fragments specific for this *Vibrio* by the PCR method without the necessity for DNA extraction or like operations from bacterial cells.

List of Sequences

Sequence No.: 1

Sequence length: 1258

Sequence type: nucleic acid

Strand number: single strand

Topology: straight chain

Sequence class: genomic DNA

Sequence

GAAGTCATCA TGACCGTTCT GCATGCCGGT GGTAAATTCG ATGATAACTC GTACAAAGTA 60

TCAGGCGGTC TTCACGGCGT GGGTGTTTCG GTAGTAAACG CACTGTCAGA AAAAGTGGTA 120

CTAACCATCC ATCGTGGCGG TCATATCCAC ACGCAAACTT ACCGTCATGG TGAGCCTGAA 180

ACGCCTCTAG CGGTTGTGGG TGATGCGGAT AAAACTGGTA CACAAATTCG TTTCTGGCCA 240

AGTGCAGAAA CTTTCTCTAA CACTGAATTC CATTACGACA TCCTAGCAAA ACGTCTGCGT 300

GAGCTATCGT TCTTGAACTC AGGCGTTTCT ATCAAGCTTA TTGATGAGCG CGAAGCGGAC 360

AAGCAAGATC ACTTCATGTA TGAAGGTGGT ATTCAAGCGT TCGTTCAGCA CTTAAACACC 420

AACAAAACAC CAATCATCGA GAAAATCTTC CACTTCGACT TAGAACGTGA AGACGGCATT 480

TCGGTAGAAG TGGCAATGCA GTGGAACGAT GGTTTCCAAG AGAACATCTT CTGTTTCACC 540

AACAACATTC CACAGCGCGA TGGTGGTACT CACCTTGCTG GTTTCCGTGC GGCAATAACA 600

CGTACGCTAA ACAGCTTTAT GGATAAAGAA GGCTTCTCGA AGAAAGCGAA AACGGCAACG 660

TCAGGCGACG ATGCGCGTGA AGGTTTGACT GCCGTTGTTT CAGTAAAAGT GCCTGATCCA 720

AAATTCTCGA GCCAAACAAA AGACAAACTG GTTTCTTCTG AAGTGAAATC AGCGGTTGAA 780

TCGGCGATGG GTGAGAAGCT ATCTGAGTTC TTGGTCGAAA ACCCAAGTGA AGCGAAAATG 840

GTTTGTTCGA AAATCATCGA TGCAGCACGT GCACGTGAAG CCGCACGTAA AGCGCGTGAA 900

ATGACTCGTC GTAAAGGCGC GCTAGACCTA GCAGGCCTAC CAGGCAAACT TGCAGACTGT 960

CAGGAAAAAG ATCCGGCACT CTCTGAACTA TACATTGTGG AGGGTGACTC TGCGGGTGGT 1020

TCAGCTAAGC AGGGTCGTAA TCGTAAGAAT CAGGCAATCC TACCACTGAA AGGTAAGATC 1080

CTGAACGTAG AAAAAGCACG TTTCGACAAG ATGTTGTCTT CGCAAGAAGT TGCAACGCTT 1140

ATTACAGCAC TTGGCTGTGG TATCGGTCGT GACGAGCACA ACCCGGACAA ACTGCGTTAC 1200

CACAACATCA TCATCATGAC CGACGCAGAC GTAGAGGCTC GCACATCCGT ACCCTGCT 1258

Sequence No.: 2

Sequence length: 419

Sequence type: amino acid

Topology: straight chain

Sequence class:

Sequence

EVIMTVLHAG GKFDDNSYKV SGGLHGVGVS VVNALSEKVV LTIHRGGHIH TQTYRHGEPE 60

TPLAVVGDAD KTGTQIRFWP SAETFSNTEF HYDILAKRLR ELSFLNSGVS IKLIDEREAD 120

KQDHFMYEGG IQAFVQHLNT NKTPIIEKIF HFDLEREDGI SVEVAMQWND GFQENIFCFT 180

NNIPQRDGGT HLAGFRAAIT RTLNSFMDKE GFSKKAKTAT SGDDAREGLT AVVSVKVPDP 240

KFSSQIKDKL VSSEVKSAVE SAMGEKLSEF LVENPSEAKM VCSKIIDAAR AREAARKARE 300

MIRRKGALDL AGLPGKLADC QEKDPALSEL YIVEGDSAGG SAKQGRNRKN QAILPLKGKI 360

LNVEKARFDK MLSSQEVATL ITALGCGIGR DEHNPDKLRY HNIIIMTDAD VEARHSVPC 419

Sequence No.: 3

Sequence length: 1258

Sequence type: nucleic acid

Strand number: single strand

Topology: straight chain

Sequence class: genomic DNA

Sequence

GAAGTCATCA TGACCGTTCT GCATGCAGGT GGTAAATTCG ACGATAACAC AAACAAATTA 60

TCGGGTGGTC TCCACGGGGT ACGTGTCTCA GTAATAAACG CACTATCAGA GAAAGTTGAG 120

CTAACGATTC ATCGTGGTGG CCATATCCAT ACGCAAACCT ACCGCCATGG TGAGCCTGCA 180

ACGCCACTAG CCGTTGTGGG TGATACGGAT AAAACCGGTA CACAAATTCG TTTCTGGCCA 240

AGTGCCGAGA CGTTCTCTAA CACTGAGTTC CACTATGACA TTCTGGCGAA ACGCCTGCGT 300

GAACTGTCAT TCCTGAACTC TGGTGTGTCG ATCAAATTGG TTGATGAACG TGAAGCGGAC 360

AAACATGATC ACTTCATGTA TGAAGGTGGT ATTCAAGCGT TCGTTGATCA CCTAAACACC 420

AACAAAACGC CAATCATCGA GAGGGTCTTC CACTTTAACT CTGAGCGTGA AGACGGCATT 480

TCAGTTGAAG TGGCGATGCA ATGGAACGAT GGTTTCCAAG AGAACATCTT CTGCTTTACC 540

AACAATATCC CACAGCGTGA TGGTGGTACT CACCTTGCTG GTTTCCGTGC TGCGCTAACA 600

CGTACATTGA ACAGCTTTAT GGATAAAGAA GGTTTCTCGA AGAAAGCGAA AACAGCGACT 660

TCAGGCGACG ATGCGCGTGA AGGTCTAACT GCGGTTGTTT CGGTGAAAGT GCCTGATCCT 720

AAGTTCTCAA GCCAAACAAA AGACAAACTG GTTTCTTCTG AAGTGAAATC AGCTGTTGAG 780

TCTGCAATGG GTGAAAAACT GTCTGAGTTC TTGATTGAGA ACCCGACAGA AGCGAAGATG 840

GTTTGTTCGA AAATCATCAA TGCAGCACGT GCATCTGAAG CAGCGCCTAA AGCTCGTGAA 900

ATGACGCGCC GTAAAGGTGC ACTAGACCTA GCAGGCCTTC CAGGTAAAGT TGCAGACTGT 960

CAGGAAAAAG ATCCGGCACT CTTTGAACTA TACATAGTGG AGGGTGAATC GGCAGGCGGT 1020

TCCGCAAAAC AAGGCCGTAA CCGTAAGAAC CAAGCGATCA CACCGCTAAA AGGTAAGATT 1080

CTTAACGTAG AAAAAGCACG TTTCGACAAG ATGCTATCTT CTCTAGAAGT AGTAACACTG 1140

ATCACCGCAT TAGGTTGTGG TATCGGTCGT GACGAGGACA ACCCCGACAA GCCTCGGGAC 1200

CACAACATAA TCATCATGAC CGACGCAGAC GTAGAGGCTC GCACATCCGT ACCCTGCT 1258

Sequence No.: 4

Sequence length: 419

Sequence type: amino acid

Topology: straight chain

Sequence class:

Sequence

EVIMTVLHAG GKFDDNTNKL SGGLHGVRVS VNALSEKVE LTIHRGGHIH TQTYRHGEPA 60

TPLAVVGDTD KTGTQIRFWP SAETFSNTEF HYDILAKRLR ELSFLNSGVS IKLVDEREAD 120

KHDHFMYEGG IQAFVDHLNT NKTPIIERVF HFNSEREDGI SVEVAMQWND GFQENIFCFT 180

NNIPQRDGGT HLAGFRAALT RTLNSFMDKE GFSKKAKTAT SGDDAREGLT AVVSVKVPDP 240

KFSSQTKDKL VSSEVKSAVE SAMGEKLSEF LIENPTEAKM VCSKIINAAR ASEAAPKARE 300

MTRRKGALDL AGLPGKVADC QEKDPALFEL YIVEGESAGG SAKQGRNRKN QAITPLKGKI 360

LNVEKARFDK MLSSLEVVIL ITALGCGIGR DEDNPDKPRD HNIIIMTDAD VEARTSVPC 419

Sequence No.: 5

Sequence length: 21

Sequence type: nucleic acid

Strand number: single strand

Topology: straight chain

Sequence class: genomic DNA

Sequence

CGG CGT GGG TGT TTC GGT AGT

Sequence No.: 6

Sequence length: 21

Sequence type: nucleic acid

Strand number: single strand

Topology: straight chain

Sequence class: genomic DNA

Sequence

TCC GCT TCG CGC TCA TCA ATA

Claims

1. An oligonucleotide having a nucleotide sequence derived from Sequence No.1, characterized in that it contains at least one site capable of amplifying a nucleotide sequence characteristic of *Vibrio parahaemolyticus*.
2. The oligonucleotide of Claim 1, having a nucleotide sequence unavailable from Sequence No.3.
3. The oligonucleotide of Claim 1 or Claim 2, incapable of amplifying nucleotide sequences originating in *Vibrio alginolyticus* and *Vibrio harvei*.
4. The oligonucleotide of Claim 1 or Claim 2 or Claim 3, represented by Sequence No.5 or Sequence No.6.
5. A method of detecting *Vibrio parahaemolyticus* by preparing a primer set comprising two of those of Claims 1 to 4, selectively amplifying therewith a DNA gyrase subunit B gene sequence contained in a specimen as a target, and determining whether or not there is a *gyrB* unit specific for *Vibrio parahaemolyticus* in the specimen.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP97/00991

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl^6 C12N15/11, C12Q1/68, C12Q1/04, C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int. Cl^6 C12N15/11, C12Q1/68, C12Q1/04, C07H21/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPI, BIOSYS, MEDLINE, GENETYX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP, 7-213299, A (Marine Biotechnology Institute Co., Ltd.), August 15, 1995 (15. 08. 95) (Family: none)	1 - 5
Y	Applied and Environmental Microbiology, Vol. 61(3)(1995) S. Yamamoto et al. "PCR Amplification and Direct Sequencing of gyrB Genes with Universal Primers and Their Application to the Detection and Taxonomic Analysis of Pseudomonas putida Strains" p. 1104-1109	1 - 5
Y	JP, 4-262799, A (Toyobo Co., Ltd.), September 18, 1992 (18. 09. 92) (Family: none)	1 - 5
Y	JP, 7-114719, B (Shimadzu Corp.), December 13, 1995 (13. 12. 95) (Family: none)	1 - 5
Y	JP, 5-123197, A (Shimadzu Corp.), May 21, 1993 (21. 05. 93) (Family: none)	1 - 5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
May 26, 1997 (26. 05. 97)	June 3, 1997 (03. 06. 97)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP97/00991

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Microbial Pathogenesis, Vol. 18 (1995) J. Okuda et al. "Distribution of the cytolethal distending toxin A gene(cdtA) among species of Shigella and Vibrio, and cloning and sequencing of the cdt gene from Shigella dysenteriae" p. 167-172	1 - 5

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